OFFICE OF NAVAL RESEARCH

CONTRACT N00014-88-C-0118

TECHNICAL REPORT 91-07

EFFECTS OF TEMPERATURE, LENGTH OF FROZEN STORAGE, AND THE FREEZING CONTAINER ON THE QUALITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

BY

C.R. VALERI, L.E. PIVACEK, A. GRAY, AND M. ERBAN

NAVAL BLOOD RESEARCH LABORATORY
BOSTON UNIVERSITY SCHOOL OF MEDICINE
615 ALBANY STREET
BOSTON, MA 02118

3 JUNE 1991

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER NBRL, BUSM 91-07	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* EFFECTS OF TEMPERATURE, LENGTH OF FROZEN STORAGE, AND THE FREEZING CONTAINER ON THE QUALITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS		5. TYPE OF REPORT & PERIOD COVERED Technical Report 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) C. Robert Valeri, Linda E. Pivacek, Alan Gray, and Melissa Erban		8. CONTRACT OR GRANT NUMBER(s) N00014-88-C-0118
9. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Blood Research Laboratory Boston University School of Medicine 615 Albany St., Boston, MA 02118		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Naval Medical Research and Development Command Bethesda, MD 20814		12. REPORT DATE 3 June 1991 13. NUMBER OF PAGES 18
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)* Bureau of Medicine and Surgery Department of the Navy Washington, D.C. 20372		15. SECURITY CLASS. *(of this report)* Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release and sale. Distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Mononuclear cells	ficoll-hypaque treatment
Blood	CFU-GEMM activity
Freezing	Plateletpheresis
Dimethylsulfoxide	

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Mononuclear cells were isolated from ficoll-hypaque treated cellular residue obtained during plateletpheresis. The peripheral blood stem cells were frozen and stored in provials or in plastic bags for as long as 2.4 years. We studied the effects of the temperature and length of frozen storage and the freezing container on the in vitro recovery and membrane integrity of the mononuclear cells in CFU-GEMM tissue culture.

The peripheral blood mononuclear cells (PBMC) from each healthy donor were

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE
S/N 0102-LF-014-6601

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

resuspended in autologous ACD plasma, and then treated with 27% DMSO in saline to achieve a final DMSO concentration of 10%. The mononuclear cells were divided among a polyvinylchloride plastic bag, a polyolefin plastic bag, and 4 polyethylene provials. The cells in the polyvinylchloride plastic bag were frozen at -80 C and the cells in the polyolefin plastic bag were frozen at -135 C; the cells in the provials were frozen at -80 C, -135 C, -150 C, or -197 C. Mechanical refrigeration was used for -80 C and -135 C storage; the vapor phase of liquid nitrogen was used for -150 C storage; and the liquid phase of liquid nitrogen was used for -197 C storage.

The mononuclear cells were frozen and stored for 1.5 years or 2.4 years, thawed, washed, and tested. The samples frozen in plastic bags in mechanical refrigerators at -80 C and -135 C for 2.4 years exhibited excellent in vitro recovery and viability values of greater than 90%. Mononuclear cells frozen and stored in plastic bags exhibited no loss of CFU-GEMM activity after 1.5 years at -80 C or -135 C; 40% loss after 2.4 years of -80 C storage; and maintenance of CFU-GEMM activity after 2.4 years of storage at -135 C.

After frozen storage at -80 C, -135 C, -150 C or -197 C for 1.5 or 2.4 years, the PBMC in provials exhibited significantly lower in vitro recovery values than the PBMC in plastic bags, although viability values werenot significantly lower. The CFU-GEMM activity was similar in the mononuclear cells frozen as long as 2.4 years in provials at -135 C, -150 C or -197 C and in plastic bags at -135 C.

EFFECTS OF TEMPERATURE, LENGTH OF FROZEN STORAGE, AND THE FREEZING CONTAINER ON THE QUALITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Valeri CR, Pivacek LE, Gray A, Erban M

ABSTRACT: Mononuclear cells were isolated from ficoll-hypaque treated cellular residue obtained during plateletpheresis. The peripheral blood stem cells were frozen and stored in provials or in plastic bags for as long as 2.4 years. We studied the effects of the temperature and length of frozen storage and the freezing container on the *in vitro* recovery and membrane integrity of the mononuclear cells, and on the growth of the mononuclear cells in CFU-GEMM tissue culture.

The peripheral blood mononuclear cells (PBMC) from each healthy donor were resuspended in autologous ACD plasma, and then treated with 27% DMSO in saline to achieve a final DMSO concentration of 10%. The mononuclear cells were divided among a polyvinylchloride plastic bag, a polyolefin plastic bag, and 4 polyethylene provials. The cells in the polyvinylchloride plastic bag were frozen at -80 C and the cells in the polyolefin plstic bag were frozen at -135 C; the cells in the provials were frozen at -80 C, -135 C, -150 C or -197 C. Mechanical refrigeration was used for -80 C and -135 C storage; the vapor phase of liquid nitrogen was used for -150 C storage; and the liquid phase of liquid nitrogen was used for -197 C storage.

The mononuclear cells were frozen and stored for 1.5 years or 2.4 years thawed, washed, and tested. The samples frozen in plastic bags in mechanical refrigerators at -80 C and -135 C for 2.4 years exhibited excellent *in vitro* recovery and viability values of greater than 90%. Mononuclear cells frozen and stored in plastic bags exhibited no loss of CFU-GEMM activity after 1.5 years at -80 C or

-135 C; 40% loss after 2.4 years of -80 C storage; and maintenance of CFU-GEMM activity after 2.4 years of storage at -135 C.

After frozen storage at -80 C, -135 C, -150 C or -197 C for 1.5 or 2.4 years, the PBMC in provials exhibited significantly lower *in vitro* recovery values than the PBMC in plastic bags, although viability values were not significantly lower. The CFU-GEMM activity was similar in the mononuclear cells frozen as long as 2.4 years in provials at -135 C, -150 C or -197 C and in plastic bags at -135 C.

INTRODUCTION: Hematopoietic stem cells isolated from peripheral blood can be used to reconstitute patients with bone marrow failure, and stem cells from peripheral blood have been recommended for hematopoietic engraftment.[1-4] Autologous peripheral blood has been used to repopulate bone marrow in cancer patients[5-10], and patients with chronic myelogenous leukemia have been treated with autologous cryopreserved leukocytes.[11-13] In studies performed at the Naval Blood Research Laboratory, mononuclear cells from human peripheral blood have been frozen, thawed, and washed, with *in vitro* recovery values of 80%.[14]

The Naval Blood Research Laboratory has studied the effects of temperature and length of frozen storage and of the freezing container on PBMC isolated from plateletpheresis residues by a ficoll-hypaque procedure[14-15]. Comparisons were made of PBMC frozen and stored in polyethylene provials at -80 C, -135 C, -150 C, and -197 C and in polyvinylchloride plastic bags at -80 C and -135 C for as long as 2.4 years. Measurements were made of freeze-thaw-wash *in vitro* recovery

and membrane integrity.[16] Pluripotential stem cell activity has been assessed in fresh and cryopreserved PBMC using the CFU-GEMM assay[17-18].

OBJECTIVE: Peripheral blood mononuclear cells were isolated from ficoll-hypaque treated plateletpheresis residue and frozen with 10% DMSO in plasma at -80 C, -135 C, -150 C, and -197 C in polyethylene provials and at -80 C and -135 C in polyvinylchloride (PVC) plastic bags. The purpose of this study was to assess the effects of temperature and length of frozen storage and of the freezing container on the *in vitro* recovery of the mononuclear cells; the membrane integrity of the mononuclear cells, and the growth of the mononuclear cells in the CFU-GEMM assay.

METHODS:

Isolation of Peripheral Blood Mononuclear cells

The mononuclear cells were isolated from the plateletpheresis residue by the Ficoll-Hypaque density (1.077 g/ml) gradient centrifugation procedure using a plastic bag system (Ethox Corp)[14-15]. The cells were washed once with 0.9% sodium chloride and were resuspended in approximately 85 ml autologous plasma.

Cryopreservation of Mononuclear Cells

The PBMC suspension was separated into aliquots which were frozen and stored in 10% DMSO and autologous plasma as follows: 2 ml volumes containing approximately 0.3 X 10^6 cells were frozen at -80 C, -135 C, -150 C or -197 C in polyethylene provials (Dynatech, Alexandria, VA); and 40 ml volumes containing approximately 6 X 10^8 cells were frozen and stored at -80 C in PL146 polyvinylchloride (PVC) plastic bags

(Fenwal Laboratories, Deerfield, IL) and at -135 C in polyolefin plastic bags (Stericon, Inc., Broadview, IL). Mechanical refrigeration was used for -80 C and -135 C storage; the vapor phase of liquid nitrogen was used for -150 C storage; and the liquid phase of liquid nitrogen was used for -197 C storage.[14]

After frozen storage for as long as 2.4 years, the PBMC were thawed and washed in a solution of 0.9 g% sodium chloride - 0.2 g% glucose - 40 mg% phosphorus, pH 5, and were resuspended in approximately 50 ml autologous plasma. The cells in the 4 provials stored at the various temperatures were thawed at the same time. The cells in the 2 PVC bags stored at -80 C and -135 C were thawed during the following week. Measurements were made of *in vitro* recovery and viability, and the recovery/viability index calculated as recovery X viability/100. CFU-GEMM assays were done to measure pluripotential stem cell activity.

In Vitro Viability Testing

The mononuclear cells were incubated with a fluoroscein diacetate and ethidium bromide mixture followed by fluorescence microscopy to evaluate membrane integrity[16].

In Vitro Assay for Hematopoietic Stem Cells (CFU-GEMM)

Two CFU-GEMM assays were employed. In the first, underlays containing human leukocytes were used. In the second, underlays without leukocytes were used and the cells were cultured in the presence of medium conditioned by leukocytes stimulated with phytohemagglutinin (PHA-LCM). Mononuclear cell preparations in a semi-solid methycellulose medium were cultured on each of the two agar-based support underlays according to a modification of the method of Ash et al[17-18].

The mononuclear cells were counted using phase microscopy, diluted with enriched ImDm-30% FBS and added to the 1.5% MC in ImDm-FBS. The final culture medium contained a known number of mononuclear cells, 1% MC in ImDm with 30% FBS, 5 X 10^{-5} M ME, and 1 unit of EP and 1 unit of heparin per ml in a total volume of 2.5 ml. Two 0.5 ml aliquots were plated into two wells of previously prepared underlays which contained 1 X 10^6/ml human leukocytes. Two additional 0.5 ml aliquots together with two 0.025 ml aliquots of PHA-LCM (final concentration of 5%) were plated on underlays containing no leukocytes. Aliquots containing 50, 70 and 100 x 10^5 PBMC were plated.

Clusters of 40 or more unfixed and unstained cells, observed by a stereoscopic microscope at 20-50X, were scored as colonies. Colonies with a reddish-white appearance were scored as mixed colonies (CFU-GEMM) containing erythroid and one or more of the following cell lines: *granulocytes, monocytes, macrophages, and megakarocytes*[17-18]. The numbers of CFU-GEMM at 50, 75 and 100 x 10^5 plating concentrations were recorded. The colonies were plotted against the plating concentration to assure a linear dose response, and the number of CFU-GEMM per 100 x 10^5 PBMC was determined from linear regression and reported.

RESULTS:

In vitro recovery and viability

Tables 1 and 2 show that the PBMC frozen and stored in provials at -80 C, -135 C, -150 C, and -197 C for as long as 2.4 years exhibited a mean post-thaw recovery value of 87 to 90%, a mean freeze-

thaw-wash recovery value of 69 to 74%, and a mean *in vitro* viability value of 86 to 92%. The mean recovery/viability index ranged from 60-65%. Slightly lower post-wash recovery and viability values were seen in the samples stored at -80 C than in those stored at -135 C, -150 C, or -197 C. When the PBMC were frozen in plastic bags at -80 C or -135 C for as long as 2.4 years, the mean post-thaw recovery values were 89 to 95%, the mean freeze-thaw-wash recovery values were 85 to 92%, and the mean *in vitro* viability values were 89 to 96%. The recovery/viability index ranged from 80 to 85% (Tables 3 and 4).

We evaluated the effects of frozen storage of PBMC in provial containers and plastic bags, and the effects of frozen storage at -80 C and -135 C, on *in vitro* recovery and viability. PBMC stored at -80 C or -135 C for 1.5 to 2.4 years exhibited significantly higher ($p<0.05$) recovery values after storage in plastic bags than after storage in provials (Table 5). The mean viability values were slightly but not significantly higher for PBMC in plastic bags than in PBMC in provials at both temperatures and at 1.5 years and 2.4 years. The recovery/viability index was significantly higher for PBMC in plastic bags than in those in provials at both 1.5 years and 2.4 years. Recovery and viability values were similar for PBMC frozen at -80 C and -135 C and in plastic bags and provials.

CFU-GEMM assay of pluripotential stem cells

After 1 to 1.5 years of frozen storage, the PBMC stored in provials showed a significantly greater decrease in pluripotential stem cell activity (CFU-GEMM) after -80 C storage than after storage at -135 C, -150 C, or -197 C (Tables 6 and 7). The CFU-GEMM assay for

PBMC in plastic bags was not significantly different after storage for 1.5 years at -80 C and at -135 C.

PBMC stored frozen at -80 C 2 to 2.4 years in either provials or plastic bags exhibited decreased CFU-GEMM activity, although the PBMC stored in provials showed the greatest reductions. PBMC stored in provials exhibited an average decrease in acitivity of 67% after thawing and 60% after thawing and washing, a statistically significant decrease ($p = 0.0001$). PBMC stored in plastic bags lost an average of 40% mixed colony formation, and showed a statistically significant decrease after washing but not after thawing.

DISCUSSION: Peripheral blood mononuclear cells obtained following ficoll-hypaque treatment of plateletpheresis residues were frozen and stored in PVC plastic bags with mechanical refrigeration at -80 C or -135 C for 1.5 years with no significant loss of pluripotential stem cell activity in 4 studies. The CFU-GEMM assays performed on PBMC after -80 C or -135 C storage were almost identical to assays done on fresh PBMC. Ten studies were completed on samples frozen in plastic bags for 2.4 years at -80 C and -135 C. After -80 C storage, thawing and washing, the PBMC exhibited a statistically significant reduction in CFU-GEMM activity of approximately 40% compared to the fresh PBMC, whereas the PBMC frozen at -135 C did not show a similar reduction in CFU-GEMM activity.

Peripheral blood mononuclear cells cryopreserved in provials at -80 C for 1.5 years showed a reduction in hematopoietic activity which was related to the -80 C storage temperature: CFU-GEMM activity after thawing and washing was significantly reduced than that seen in PBMC

frozen and stored at -135 C, -150 C, and -197 C. After 2.4 years of frozen storage at -80 C, a similar reduction was observed.

The mean PBMC *in vitro* freeze-thaw-wash recovery value after storage in plastic bags at -80 C and -135 C for as long as 2.4 years was greater than 90%, and the mean viability value was greater than 94%, significantly higher than the PBMC stored in provials. The mean freeze-thaw-wash recovery values of PBMC frozen in provials at -80 C or -135 C and stored for 1.5 years and 2.4 years were less than 75%. The viability values were also slightly but not significantly lower in the PBMC frozen and stored in provials than in those stored in plastic bags.

PBMC stored frozen in plastic bags for 1.5 to 2.4 years showed significantly higher recovery, viability and pluripotential stem cell activity than PBMC stored in provials. These data indicate that the quality of the PBMC frozen in plastic bags cannot be determined from values obtained from PBMC frozen and stored in provials.

Peripheral blood mononuclear cells stored in polyvinylchloride plastic bags at -80 C and -135 C with mechanical refrigeration for as long as 2.4 years have exhibited excellent *in vitro* recovery and viability. The growth of the PBMC in the CFU-GEMM assay was maintained following storage at -135 C for 2.4 years and moderately reduced following storage at -80 C for 2.4 years.

REFERENCES

1. Barr R, Stevens C, Koekebakker M, McBride J: Collection of erythroid progenitor cells by cytapheresis of peripheral blood of normal donors. Transfusion 22:388-391, 1982.

2. Deisseroth A, Abrams R: The role of autologous stem cell reconstitution in intensive therapy for resistant neoplasms. Cancer Treatment Rep 63:461-471, 1979.

3. Fliedner T, Calvo W, Korbling M, Nothdurft W, Pflieger H, Ross W: Collection, storage and transfusion of blood stem cells for the treatment of hemopoietic failure. Blood Cells 5:313-328, 1979.

4. Gale R, Champlin R, Feig S, Fitchen J: Aplastic anemia: biology and treatment. Ann Intern Med 95:477-494, 1981.

5. Kessinger A, Armitage JO, Landmark JD, Weisenburger DD: Reconstitution of human hematopoietic function with autologous cryopreserved circulating stem cells. Exp Hematol 14:192-196, 1986.

6. Korbling M, Dorken B, Ho AD, Pezzutto A, Hunstein W, Fliedner TM: Autologous transplantation of blood-derived hemopoietic stem cells after myeloablative therapy in a patient with Burkitt's lymphoma. Blood 67:529-532, 1986.

7. Reiffers J, Bernard D, David B, Vezon G, Sarrat A, Marit G, Moulinier J, Broustet A: Successful autologous transplantation with peripheral blood hemopoietic cells in a patient with acute leukemia. Exp Hematol 14:312-315, 1986.

8. Kessinger A, Armitage JO, Landmark JD, Smith DM, Weisenburger DD: Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. Blood 71:723-727, 1988.

9. Kessinger A, Armitage JO, Smith DM, Landmark JD, Bierman PJ Weisenburger DD: High-dose therapy and autologous peripheral blood stem cell transplantation for patients with lymphoma. Blood 74:1260-1265, 1989.

10. Kessinger A, Armitage JO: The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies (editorial). Blood 77:211-213, 1991.

11. Goldman J: Modern approaches to the management of chronic granulocytic leukemia. Semin Hematol 15:420-430, 1978.

12. Goldman J: Autografting cryopreserved buffy coat cells for chronic granulocytic leukemia in transformation. Exp Hematol 7:389-397, 1979.

13. Korbling M, Burke P, Braine H, Elfenbein G, Santos G: Successful engraftment of blood derived normal hemopoietic stem cells in chronic myelogenous leukemia. Exp Hematol 9: 684-690, 1981.

14. Isolation, Purification, Freezing, Thawing, Washing, and Resuspension of Mononuclear Cells Isolated From Bone Marrow and from Peripheral Blood: Technical Report 89-12 NBRL-BUSM.

15. Carciero R, Valeri CR: Isolation of Mononuclear Leukocytes in a Plastic Bag System Using Ficoll-Hypaque. Vox Sang 49:373-380, 1985.

16. Lionetti FJ, Hunt SM, Lin PS, Kurtz SR, Valeri CR: II Characteristics of Granulocytes Obtained By Counterflow Centrifugation: Transfusion 17:465-472, 1977.

17. Ash, RC, Detrick, RA, and Zanjani, ED: Studies of Human Pluripontial Hematopoietic Stem Cells (CFU-GEMM) in Vitro. Blood 58:309, 1981.

18. O'Murchadha E, Horland A, Neiman R, and Valeri CR: Morphology of cells grown in CFU-GEMM tissue culture assay from mononuclear cells obtained from peripheral blood and bone marrow of normal volunteers. Exp Hematol 16:235-239, 1988.

TABLE 1

RECOVERY FOLLOWING LONG TERM FROZEN STORAGE OF PBMC IN PROVIALS

Length of fzn Storage	-80 C	-135 C	-150 C	-197 C
Thaw Recovery	**1.5 Yrs**			
Mean:	88.58	89.92	86.83	88.58
SD:	8.55	7.65	11.04	9.32
n:	12	12	12	12
	2.4 Yrs			
Mean:	89.58	88.25	90.42	90.36
SD:	7.88	9.31	9.12	8.36
n:	12	12	12	11
Wash Recovery	**1.5 Yrs**			
Mean:	79.83	82.42	82.75	81.91
SD:	7.88	9.31	9.12	8.36
n:	12	12	12	11
	2.4 Yrs			
Mean:	79.83	80.92	79.08	79.00
SD:	9.24	10.81	10.14	9.44
n:	12	12	12	11
Freeze-Thaw-Wash Recovery	**1.5 Yrs**			
Mean:	70.67	73.75	71.58	73.00
SD:	11.42	10.14	9.83	9.69
n:	12	12	12	11
	2.4 Yrs			
Mean:	69.25	73.50	70.58	71.64
SD:	8.61	8.80	9.07	6.95
n:	12	12	12	11

TABLE 2

VIABILITY AND RECOVERY/VIABILITY INDEX FOLLOWING LONG TERM FROZEN STORAGE OF PBMC IN PROVIALS

Length of fzn Storage	-80 C	-135 C	-150 C	-197 C
Viability 1.5 yrs				
Mean:	85.67	86.33	89.75	88.82
SD:	7.90	6.43	5.07	5.56
n:	12	12	12	11
2.4 yrs				
Mean:	88.17	88.25	91.92	90.64
SD:	4.13	6.06	3.23	3.59
n:	12	12	12	11
Recovery/Viability Index Viable 1.5 yrs				
Mean:	60.25	63.58	65.08	64.18
SD:	9.62	10.05	10.12	10.80
n:	12	12	12	11
2.4 yrs				
Mean:	62.42	65.00	65.17	64.82
SD:	8.22	9.52	10.00	6.40
n:	12	12	12	11

TABLE 3

RECOVERY FOLLOWING LONG TERM FROZEN STORAGE OF PBMC IN PL146 POLYVINYLCHLORIDE PLASTIC BAGS STORED AT -80C AND IN POLYOLEFIN PLASTIC BAGS STORED AT -135C

	Time Frzn	-80 C	-135 C
Thaw Recovery	**1.5 yrs**		
Mean:		92.67	89.00
SD:		11.31	6.52
n:		6	5
	2.4 yrs		
Mean:		93.80	95.00
SD:		8.01	8.55
n:		10	10
Wash Recovery	**1.5 yrs**		
Mean:		97.50	96.83
SD:		6.12	5.15
n:		6	6
	2.4 yrs		
Mean:		98.30	94.80
SD:		5.03	9.75
n:		10	10
Freeze-Thaw-Wash Recovery	**1.5 yrs**		
Mean:		90.17	85.40
SD:		11.02	6.50
n:		6	5
	2.4 yrs		
Mean:		92.20	90.20
SD:		9.86	11.23
n:		10	10

TABLE 4

VIABILITY AND RECOVERY/VIABILITY INDEX FOLLOWING LONG TERM FROZEN STORAGE OF PBMC IN PL146 POLYVINYLCHLORIDE PLASTIC BAGS STORED AT -80C AND IN POLYOLEFIN PLASTIC BAGS STORED AT -135C

	Length of fzn Storage	-80	-135
Viability	**1.5 yrs**		
Mean:		94.33	95.50
SD:		2.16	1.38
n:		6	6
	2.4 yrs		
Mean:		91.09	88.82
SD:		6.98	6.51
n:		11	11
Recovery/Viability Index	**1.5 yrs**		
Mean:		85.17	82.40
SD:		10.01	4.98
n:		6	5
	2.4 yrs		
Mean:		83.60	80.20
SD:		10.28	12.50
n:		10	10

TABLE 5

ANALYSIS OF VARIANCE (ANOVA)

Recovery and In Vitro Viability Measurement of Frozen, Thawed, Washed PBMC

1. Effect of Freezing Container, Provials vs Plastic Bags Frozen at -80C or -135 C

Frozen Storage	MEAN VALUES Provials -80 C	Provials -135 C	Bags -80 C	Bags -135 C	ANOVA Provials vs Bags
1-1.5 yrs					
Thawed Recovery	88.6	89.9	92.7	89.0	NS
Wash Recovery	79.8	82.4	97.5	96.8	p <0.05
Thaw-wash Recovery	70.7	73.8	90.2	85.4	p <0.05
Viability	85.7	86.3	94.3	95.5	NS
Recovery/ Viability Index	60.3	63.6	85.2	82.4	p <0.05
2-2.4 yrs					
Thawed Recovery	89.6	88.3	93.8	95.0	NS
Wash Recovery	79.8	80.9	98.3	94.8	p <0.05
Thaw-wash Recovery	69.3	73.5	92.2	90.2	p <0.05
Viability	88.2	88.3	91.1	88.8	NS
Recovery/ Viability Index	62.4	65.0	83.6	80.2	p <0.05

2. Effect of Temperature of Storage

A) No significant effect of temperature on recovery or viability of samples frozen in provials or plastic bags.

TABLE 6

CFU/GEMM ASSAY OF HUMAN MONONUCLEAR CELLS AFTER FROZEN STORAGE FOR AS LONG AS 2.3 YEARS AT -80, -135, -150, AND -197 C IN PROVIALS AND PLASTIC BAGS

CFU/GEMM Colonies per 100,000 MNC plated (average of cellular underlay and PHA/LCM methods)

			PROVIALS								BAGS			
FROZEN		WITH	THAWED				WASHED				THAWED		WASHED	
STORAGE	FRESH	DMSO	-80C	-135C	-150C	-197C	-80C	-135C	-150C	-197C	-80C	-135C	-80C	-135C
1.5 yrs														
Mean:	19.0	18.5	12.0*	17.9	16.9	18.9	12.8*	18.4	18.1	17.9	16.3	16.8	15.0	15.8
SD:	7.9	8.0	8.5	7.3	7.9	8.8	10.4	10.4	6.7	8.3	2.2	5.3	2.9	6.1
n:	10	10	8	8	8	8	8	8	8	8	4	4	4	4
2.4 yrs														
Mean:	20.9	22.5	7.4*	15.0	23.3	21.0	8.4*	19.8	20.6	18.7	19.5	20.1	12.9*	19.2
SD:	7.6	7.8	6.6	7.5	9.8	9.3	5.0	8.7	9.3	6.5	10.6	16.1	8.6	12.5
n:	11	11	11	11	11	11	11	11	11	10	10	9	10	9

*p <0.05 analysis of variance showed significant temperature effect.

TABLE 7

ANALYSIS OF VARIANCE (ANOVA)

CFU-GEMM Assay of Pluripotential Stem Cell Activity

Effect of temperature of storage.
ANOVA performed on samples thawed and washed on the same day:

1. Provials at 4 temperatures.
2. Bags at 2 temperatures.

	Mean CFU-GEMM/100,000 PBMC					
	1. Provials,				2. Bags	
	-80 C	-135 C	-150 C	-197 C	-80 C	-135 C
A. 1-1.5 yrs frozen, n=8					n=4	
Thawed	12.0+	17.9	16.9	18.9+	16.3	16.8
Washed	12.8*	18.4	18.1	17.9	15.0	15.8
B. 2-2.4 yrs frozen, n=11					n=10,	9
Thawed	7.4*	15.0	23.3	21.0	19.5	20.1
Washed	8.4*	19.8	20.5	18.7	12.9*	19.2

+ $p<0.05$, compared to 1 other temperature group.
* $p<0.05$, compared to all other temperature groups.